Metabolically engineered organisms could sustainably produce ingredients for natural foods, flavors and fragrances

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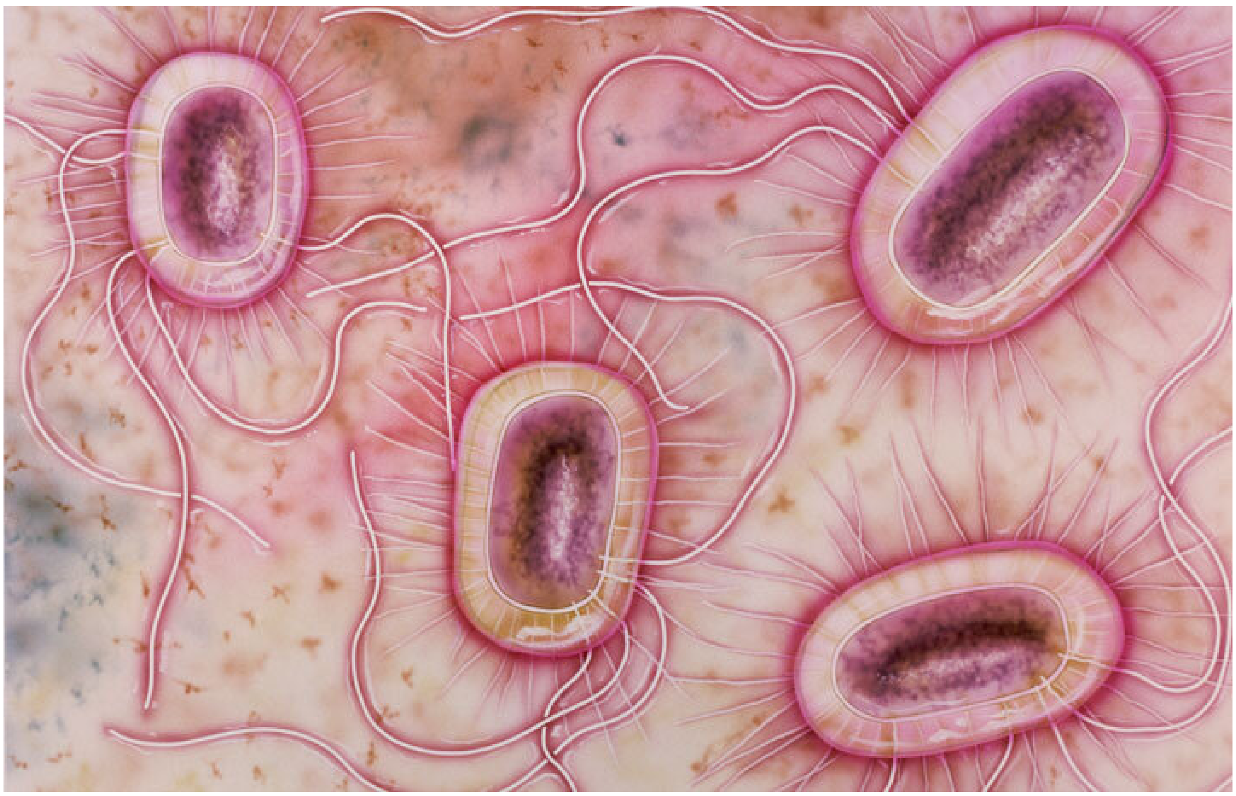


Escherichia coli bacteria are Gram-negative, rod-shaped bacteria that normally inhabit the intestines of humans and other animals. They can also produce 'natural-identical' compounds, unlike synthetic and chemical production methods. Credit: RBO VEISLAND, MI&I/Science Photo Library metabolically engineered organisms could sustainably produce ingredients for natural foods, flavors and fragrances

Is that floral scented, natural shampoo from the health foods store good for the planet? Demand for organic food and fragrance is growing, but harvesting the ingredients used in natural products is putting a strain on limited environmental resources. One solution is to turn to microscopic organisms that produce 'natural' ingredients in vats of low-value fermented feedstock.

Microbes produce 'nature-identical' compounds. While synthetics are a mixture of isomers, which have a similar structure to naturally occurring compounds, they aren't quite the same, explains A*STAR microbiologist Nic Lindley. In addition, a few tweaks to the metabolisms of the right microscopic organisms can power industrial-level production that often outperforms chemical synthesis or extraction in terms of production rates and cost-effectiveness.

Sharp output increases from yeast and bacteria are already being recorded at the BioTransformation Innovation Platform (BioTrans), the part of A*STAR's Food, Nutrition and Consumer Care (FNCC) cluster focused on compounds for the health, seasonings and personal care sectors.

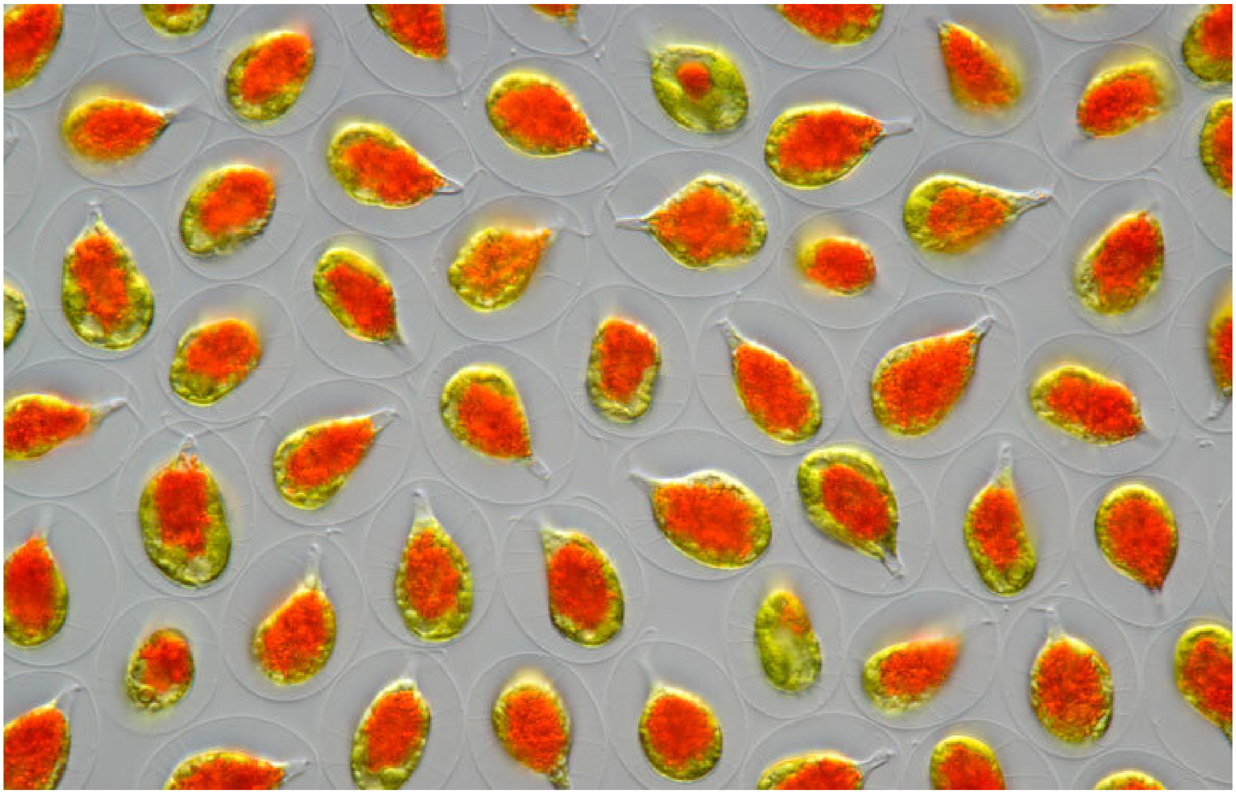
Lindley moved to Singapore from France two years ago to lead the platform. Since its launch in 2016, BioTrans has focused on five goals for the future of food and fragrance, says Lindley. These aims are to:

- Maximize product yields through its Microbial Metabolism Platform;
- Accelerate product development to get products to market faster via its Fermentation and Downstream Platform
- Discover new compounds, biosynthetic pathways and microbial

strains by trawling A*STAR's vast Natural Product Library
- Advance the development of healthier sweet, savoury and other flavorings as part of the BioTrans Taste Receptor Platform
- Investigate flavors, fragrances, and metabolites by combining mass spectrometry with organoleptics (the study of sensory perception) to strengthen its Analytical Platform

The sweet smell of success

Improvements in microbe production rates have steadily emerged from the BioTrans Microbial Metabolism Platform. In 2017, after increasing the output of a lycopene-overproducing E. coli strain, researchers reported unprecedented yields of two high-demand natural aroma compounds—alpha-ionone, known for its sweet, violet-like aroma, and beta-ionone, which produces a scent often associated with violet or raspberry. On the upper end, BioTrans researchers beat a yeast's proven beta-ionone production rate, improving output 80-fold using E. coli.



A light micrograph of the resting, or cyst, phase of Haematococcus pluvialis chlorophytes, a type of green algae. They contain the pigment astaxanthin (red), an antioxidant and important cosmetics ingredient that is currently expensive to produce. BioTrans researchers used E. coli to improve on the production rate of algae by a factor of 16. Credit: WIM VAN EGMOND/Science Photo Library

The two ionones are used in everything from perfumes and oils to ice cream and maraschino cherries. Lycopene, the carotenoid pigment that makes tomatoes red, is one of the main precursors of these types of apocarotenoids. Carotenoids have global market worth that is expected to reach USD 1.53 billion dollars by 2021.

The complex metabolic characteristics of apocarotenids have been a challenge to produce in the laboratory. In their quest to optimize output, the BioTrans team divided E. coli's apocarotenoid producing biosynthetic pathway into four sections, or 'modules', and optimized enzymatic steps in each through genetic and enzyme engineering.

The group describe their method as a 'plug-and-play' system, meaning that "the platform is capable of producing various apocarotenoids simply by changing one or two genes, without perturbing the whole strains," says lead author Simon Congqiang Zhang, a BioTrans research fellow, who supervises several translational research projects. This system has been shown to also work on a number of similar substances, including lycopene, beta-carotene, phytoene and retinol (vitamin A), an ingredient that will be familiar to those who use wrinkle creams or acne treatments (see our recent story on acne).

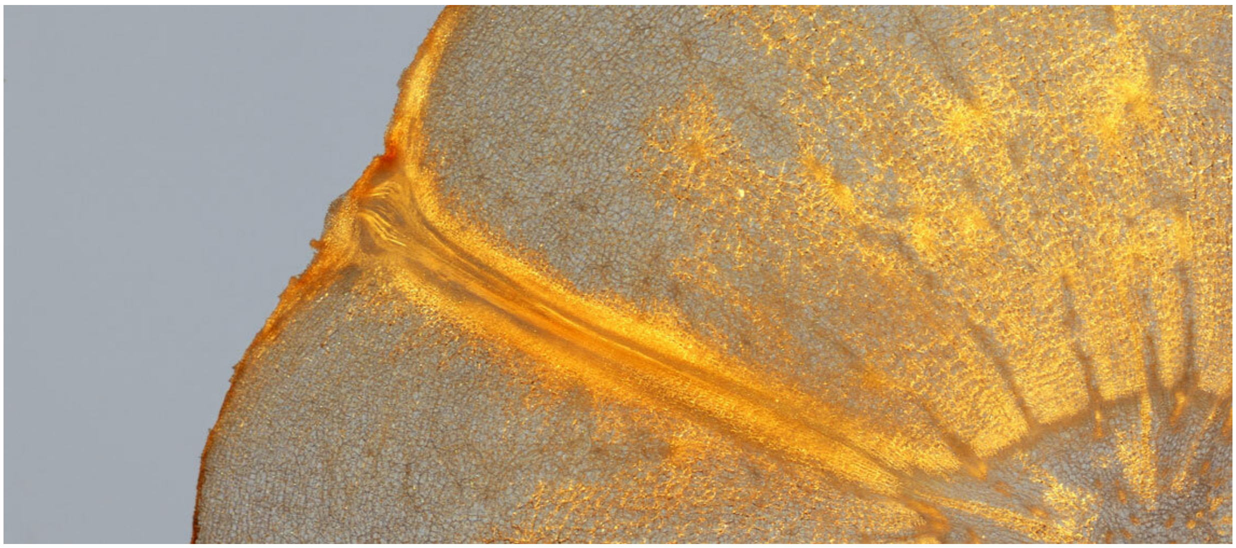
Patenting the system is the next step, and Zhang reports that there are "quite a few companies in discussions with us to co-develop the bioprocess for commercialisation". Meanwhile, BioTrans scientists are also examining whether product range development can be pursued internally, with a view to spinning out their own commercial venture.

Accelerated antioxidants

In 2018, Zhang led another study building on his apocarotenoid work. The group used E. coli to produce a 1600 per cent improvement on current methods for producing the antioxidant, astaxanthin, another type of carotenoid, and the red pigment that makes salmon and shrimp pink.

Astaxanthin exhibits strong antioxidant, anti-inflammatory, and anti-cancer activity, and Zhang points out that its biological activities and benefits are supported by more than 50 human clinical trials and more than 1,000 papers published in leading journals. As a result, it is widely used in food, feed, nutraceuticals, cosmetics and medicine. The global astaxanthin market is predicted to reach USD 2.57 billion by 2025.

Most of the astaxanthin produced commercially is derived from algae such as Haematococcus pluvialis. This freshwater, unicellular green microalga transforms into red cysts under stress conditions, including nutrient deficiency, salinity, and high temperatures, in combination with high irradiance.



A light micrograph of a thinly sliced section of carrot rich in the chemical beta-carotene, which the body converts into retinol (vitamin A). Natural retinol is usually produced using plants that contain beta-carotene, a red-orange pigment. But beta-carotene can also be produced by E. coli, and this production is being tweaked for possible commercial applications by BioTrans researchers. Credit: Eye of Science/Science Photo Library

But the algae is slow growing and usually produced in closed clear tubes (photobioreactors) to reduce cross-contamination from microorganisms such as microalgae, fungal parasites, and zooplankton predators. As a result, producing astaxanthin requires a relatively large amount of land, and costs more than USD 7,000 per kilogram.

By building on the same modular approach used for apocarotenoids, the team from BioTrans and the National University of Singapore, developed a complex self-learning system, known as a multidimensional heuristic process (MHP), capable of balancing the enzymatic activity within and among different modules simultaneously.

Using this approach, in *Nature Communications*, the team reported a nature-identical astaxanthin production rate that was 16 times higher than the algae-based production rate, with an enantiomeric excess (a measure of purity) of 100 percent.

"The main advantage of the MHP is it enables the systematic, but rapid optimization of biological systems, to achieve high yield production of metabolites [such as astaxanthin]," Zhang explains.

This system is now on the cusp of commercialization. "Our current technology is able to produce astaxanthin at high concentrations and low cost on a small scale," says Zhang. "The next challenge is to scale up our process in large fermenters, with a capacity of up to ten cubic metres, and purify the compounds effectively."

Zhang also points out that the team's MHP has been used to optimize many different metabolic pathways and molecules. Used with mathematical models, it could improve the performance of many microbes, he says. It has already been applied to produce of nerolidol, well known for its fresh, woody scent and frequently used in shampoos, body lotions and soaps, and linalool, a compound with citrus tones used in many cleansing lotions, aftershaves and hair care products. With the MHP, E. coli produced linalool at 680 times the rate of a previously engineered yeast strain.

Searching a trove of tastes

BioTrans researchers are also on the hunt for new food ingredients. Over at the BioTrans Taste Receptor Platform, senior research fellow Ann Koay and her colleagues mine A*STAR's rich resources for molecules that refine taste sensations—work that might provide people with more options so that they can make healthier choices.

BioTrans researchers use cell systems that mimic the human tongue to quickly assess the qualities of flavour molecules. Credit: BioTransformation Innovation Platform

To find these molecules, Koay trawls A*STAR's Natural Product Library (NPL), one of the world's largest resources for the study of biotechnologically useful microorganisms, genes, enzymes and other bioactive compounds. Established in 2013, the NPL houses more than 37,500 plant samples, 123,000 microbial strains, and 270,000 extracts derived from these specimens, collected from more than 100 countries. According to researchers at A*STAR's Bioinformatics Institute, who manage the NPL, the collection represents "57 percent of all known cultured fungal genera, 67 percent of the world's plant families, and 70 percent of filamentous bacterial genera." Genome sequence data for more than 150 microbial strains at NPL have already been uncovered, with potentially thousands more in the pipeline, and plans are underway to develop a digital database that will enable researchers to probe microbial genomes in even greater detail.

To make use of this huge resource, BioTrans researchers have developed cell systems that mimic human taste responses to sweet and bitter tastes, enabling researchers to quickly evaluate thousands of molecules for their taste-modulating properties. Cell systems are faster and cheaper than human sensory panels (groups of people who give feedback on taste and sensation). By further improving these high-throughput screening approaches, Koay hopes to accelerate discoveries of novel molecules, adding to the pipeline of molecules that are safe, healthy, and effective for use in the food industry.

Of particular interest are molecules to help address increasing rates of diabetes. Half of Singapore's population will have type 2 diabetes by 2050, according to a multi-institutional study published in 2014. To combat this disease, an increasing number of food companies are focusing on sugar reduction or replacing sugar in their products with sugar alternatives and sweet taste enhancers. The latter are molecules that increase the sweet taste sensation but do not taste sweet themselves. Even small amounts of such molecules can potentially replace or reduce the large amounts of table sugar currently added to processed foods, says Koay.

Koay and her colleagues are also looking at the other side of the coin, setting up a screening panel of bitter taste tests to search the library for bitterness blockers. These can enhance naturally sweet flavors by blocking the interaction between bitter compounds and human taste receptors.

Sensations such as cooling, warming, and tingling are also important to consumers says Koay. People perceive quality and effectiveness of products from these sensations, so the researchers want to examine "sensates" —molecules that correspond. These can send important underlying signals to consumers, who associate coolness with cleanliness for example, or hotness with active ingredients. New and more potent ingredients—cooling agents for example—can be utilized to improve products for the consumer care industry, Koay explains.

Researchers at the BioTrans Analytical Platform are already using mass spectrometry with organoleptics (the study of sensory perception) to look at the chemical compositions that affect the sensory aspects of flavors, fragrances and metabolites. Koay and researchers at the Taste Receptor Platform want to expand their repertoire of cell system tests to examine sensates for thermoreception and chemical sensitivity.

Once useful molecules are found and demonstrated to be safe and scalable, Koay says, then commercial level production can be assessed by colleagues, such as Zhang.

Microbe engine for industry

While synthesis is still the main source of chemicals for the food and fragrance industries, Zhang firmly believes that in the future, "biosynthesis will be on par with chemical synthesis". Meanwhile, he will continue to focus on three main areas: the discovery of new enzymes and novel metabolites, such as terpenoids (the aromatics used in many scents); enzyme engineering for improved activity, stability and selectivity; and building on fundamental understandings of biosynthesis.

Lindley agrees that there are big changes ahead. He says that even capability-building projects, such as their work on lycopene, always aim for twin outcomes: "to expand our knowledge base and technological toolbox, and also to search for molecules applications that we believe will have industrial potential."

But the biggest challenge has moved quickly to implementation. The conditions used in industrial processes are often far from the evolutionary pressure that has guided microbial evolution over millions of years, says Lindley. The platform will be carefully evaluating "how robust our metabolically engineered microbes will be when we transfer them into industrial-scale fermentation strategies." It means the hard science at BioTrans must be supported by practical industrial considerations, he says, including rational projections and responsive development strategies. The team will also seek to use industrial side-streams by converting by-products and wastes into other, value-added products.

BioTrans, says Lindley, already has an edge in understanding and being able to exploit areas in which few laboratories have experience. "BioTrans was conceived as an industry-facing initiative, able to interact directly and resolve company problem statements efficiently and rapidly," he says. "This gives us a clear competitive edge when we are discussing, with companies, their ambitious plans to put novel molecules on the market."

Singapore, he notes, is also ideally placed "to position itself as the hub for R&D aimed at the Asian consumer, and offers the opportunity to develop new molecules that may not be part of plans in the US or Europe." As a result, a new wave of Singapore-made, microbe-aided products are likely to hit the shelves in the near future.

"We have most of the world's leading food and cosmetics ingredients producers within a few minutes' drive from the lab and an equally rich diversity of nearby final end-user brand names," notes Lindley. Most, he says, are positioning themselves to service a growing middle class both in Asia and around the world, populations increasingly able to access information about the impacts of the ingredients they use, and willing to invest in healthy, sustainable and natural products.

More information: Congqiang Zhang et al. A "plug-n-play" modular metabolic system for the production of apocarotenoids, *Biotechnology and Bioengineering* (2017). DOI: 10.1002/bit.26462

Congqiang Zhang et al. Multidimensional heuristic process for high-yield production of astaxanthin and fragrance molecules in Escherichia coli, *Nature Communications* (2018). DOI: 10.1038/s41467-018-04211-x

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